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14. ABSTRACT
This one year Exploration-Hypothesis Development Award is aimed to identify the possible molecular markers to predict the malignant tendency of the Prostate Intraepthelial Neoplasia (PIN) lesion. During the past year, our main effort has been made to establish reliable methods to micro-dissect the PIN cells, to extract RNA, and to use this RNA to perform microquantity differential display to analyze the differential expression patterns between the cancer precursor PINs and the benign PIN lesions. Altogether, we have assessed about 5000 functional genes and found 53 differentially-expressed bands. With the limited time schedule, we have tried to recover 11 genes and successfully obtained 7 sequences. We have examined the expression status of these 7 candidates in a large number of PINs and in an archival set of prostate tissues. While continue to assess their potential of predicting malignant tendency of PINs, we have also found that one of them (osteopontin) is an important prognostic factor for prostate cancer.

15. SUBJECT TERMS
Prostate Intraepithelial Neoplasia, Molecular Markers, Malignant Progression, Prostate Cancer, and Benign Prostate Lesions

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Introduction

This report is the final report for a one-year project titled "identification of possible molecular markers to predict the malignant tendency of the prostate intraepithelial neoplasia (PIN) lesions", which was supported by an Exploration-Hypothesis Development Award for the DOD PCRP grant. Prostate Intraepithelial Neoplasia (PIN) is a pathological lesion that can either progress to prostate cancer or remain to be a benign prostatic lesion. Currently, there is no method to predict which PINs will become cancer and which will not. Through analysing the gene expression patterns between the PINs of different natures, it may be possible to find out some molecular markers that can be used to predict the malignant tendency of PINs. Prostate cancer is usually heterogeneous and multifocal with diverse clinical and morphologic manifestations. Currently, Prostate Intraepithelial Neoplasia (PIN) is the only identifiable precursor lesion. The genetic similarities between PIN and the metastatic cancer strongly suggest that evolution and clonal expansion of PIN may involve important molecular events that lead to the onset and development of prostate cancer [1, 2]. Thus PIN is the earliest accepted and morphologically identifiable stage in prostatic carcinigenisis, possessing many of genotypic changes of cancer [1, 2]. Although some PINs will progress to cancer in a relatively short period of time, others always remain to be non-malignant and never become cancer [2, 3]. Thus, currently the PINs capable of becoming cancer cannot be distinguished from those incapable of becoming cancer by morphological means. There are no available methods to decide which PIN remains stable, which PIN progresses or regresses, although the implication is that it can progress. Because of this difficulty, when and how to treat PINs remains to be a controversial issue [4]. It is hypothesized that it may be the occurrence of the relevant genetic changes that will decide whether or not a PIN is a cancer precursor. This hypothesis is supported by the recent finding that some of the PINs shared cytogenetic alterations with invasive prostate cancer [5] and that the cancer-precursor PINs possessed many genotypic changes of the cancer [6-8]. Although the pre-occurred genetic changes within the cancer-precursor PINs responsible for their malignant progression may involve in a series of complicated sequential genetic events, these changes can be classified in a simple way as the increased activity of the carcinogenisis-promoting genes and the decreased activity of the possible tumour-suppressor genes. This project is aimed to identify the major genes involved in malignant progression and to study how they work within the precursor cells and thus to find possible markers to predict the malignant tendency of the PINs so that the cancer precursor can be identified and treated at the pre-maligant stage. We planned perform the investigation by using our recently developed micro-quantity differential display (MDD) approach [9-11] which can be used to systematically analyse differential- gene- expression with as little as 2.5ng starting total RNA material. Our initial target outline in our original grant proposal is test the feasibility and this research by analysing some subsets of mRNA form PIN tissues and to identify at least one candidate gene that is differentially expressed between the two different types of PINs.

Body of the Proposal

According to our original plan, the objective of this preliminary study is to test feasibility of the proposed work by searching for some possible molecular markers that can be used to distinguish the cancer-precursor PINs from the benign PIN lesions through analysing the differential expression of a number of subsets of RNA between the two PIN types. It was our hope that this work would produce sufficient preliminary data to enable us to apply for a three-year standard research project grant to assess systematically the differential gene expression profiles between the two PIN types and to identify and characterise the possible

genes involved in the initiation of the malignant progression of prostate cancer at the early stage.

Our first task proposed in the original plan is to identify some differentially expressed genes between the cancer-precursor PINs and the non-precursor PINs. As described in the plan, we had spent one month to re-examine an archival set of paraffin-embedded radically-prostatectomy specimens collected between 1992-1998 (about 1000 cases) from clinical departments through our diagnostic pathology laboratory to identify all cases containing PIN areas with the criteria of McNeal and Bostwick [12]. The cancer precursor PINs and non-precursor PINs were identified according to the record results of biopsies performed within the subsequent 3 years. Dr. Shiva Forootan examined a very large number of cases and selected about 600 PINs. But when she checked following up records, she found that most of the PINs were taken together with carcinomas and are cancer-precursors. She only obtained about 100 PINs that are non-precursor benign lesions. Although we have not got equal numbers for both PIN types and only had about 100 benign PINs, we believed that this was sufficient to perform the proposed preliminary investigation. However, if this project is to be continued in the future, it may be necessary that we shall obtain some more benign PINs.

During the second and third month of study, our effort had been focused on isolation of the PIN cells by micro-dissection and on extraction of RNA from these epithelial cells. Laser Capture Micro- dissection method was used to dissect about 300 cells from each PIN area. To avoid the possible clonal variations, cells dissected from 6 areas of cancer-precursor and non-precursor PINs were mixed respectively to form two separate collections respectively. The total RNA from these two cell collections were extracted respectively with an Inviscreen RNAce Reagent Kit according to the manufacturer's instruction. This kit was developed recently by BIOLINE (UK) specifically for extraction of small amount of RNA. According to our estimation, we had obtained about 100ng of RNA from each of the cell collections.

We used the RNA to perform analyze the differential expression profiles between the types of PINs through the MDD technique. As originally planed, we have displayed 50 of the 192 subsets of the RNA and repeated the display twice. Assuming that each display gel contained about 100 cDNA fragments, we had analyzed about 5,000 mRNA species. During the three rounds of repeated MDD analyses, there were 53 bands exhibited a consistently more than 2-fold differences. The most pronounced differences were observed in three bands. When we recovered these most differentially expressed cDNA fragments from the denaturing gel and to analyse their nucleotide acid sequences, it was found that they represented the following three genes: the gene coding for the secreted protein osteopontin (OPN) [13], the tumour-suppressor gene teazrotene induced gene 1, or TIG1 [14], and the gene coding for the calcium-binding protein S100A4 [15]. All these three genes had been previously implicated either in prostate cancer or in cancers of other locations. With the limited time schedule, we had recovered 11 of the 53 bands and successfully sequenced 7 of the 11 recovered molecules.

The work described in task 1 of the original plan (Statement of Work) was finished in 7.5 months, slightly ahead of the original schedule.

The second phase of this study, as outlined in ***Task 2,*** is to verify the differences of the candidate genes through assessing the levels of their mRNA by *in situ* hybridisation (when antibody was not available) or immunohistostaining (when antibody is available) in large

numbers of PINs and to assess the potential of each candidate as a molecular marker. We spent only 4.5 months to examine the expression status of the 7 of the 53 candidate genes in 300 cancer precursor PINs and in 100 non-precursor PINs. The reason for the speedy completion of this part of the work is that the antibodies against all candidates, but TIG1, were already available and thus we can use immunohistostaining to assess 6 candidates. The *in situ* hybridization analysis was performed only with TIG1 cDNA. We had used statistical analyses to carefully assess the potential of each of the candidate genes as molecular markers to distinguish the two types of PINs. In addition, we had extended the immohistological analysis of several of the identified proteins to a large number of benign prostate hypoplasia (BPH) and prostate carcinoma tissues. Their expression status and the prognostic significance for patient outcome were also systematically assessed.

Our results showed that amongst the 7 candidate genes detected by MDD, at least 2 (osteopontin and S100A4) exhibited significantly different expression patterns between the cancer-precursor PIN tissues and the benign PIN tissues. Amongst the 300 cancer-precursor-PINs, all (100%) expressed OPN. Amongst the 100 benign PINs, 88 (%) expressed OPN. Although the percentages of the cases expressed OPN was not significantly different, the difference between the stain intensities of the two types of PINs was highly significant (Chi-Square test, $P < 0.007$). In the same set of the samples, 101 (33.6%) of the 300 cancer-precursor PINs expressed S100A4, whereas none (0%) of the benign PINs expressed it. The difference was highly significant (Chi-Square test, $P < 0.001$). OPN exhibited different expression patterns between the two types of PINs. Although we did not found a clear cut relationship between the elevated level of OPN and the malignant tendency of PIN, we did find that increased OPN expression was an important prognostic marker to predict the patient survival [14]. As to the S100A4, it was expressed in 33% of the cancer-precursor PINs, but not in any of the benign PINs, its expression appeared to indicate a malignant progression. However, only a small proportion of the cancer precursor PINs expressed S100A4, it can not be used as an indicator for those cases where S100A4 is not expressed. More work is need for further assessment of the possibility of using S100A4 and a molecular marker to predict the malignant tendency of PINs.

Key Research Accomplishments

In the project, we have made the following key research accomplishments:

1. Successfully isolated prostate epithelial cells for PIN lesions and extracted RNA.

2. With these RNAs, we have successfully performed MDD analysis to assess the expression patterns of different PIN lesions.

3. We have assessed about 5,000 cDNA bands with MDD and have identified 53 differentially expressed bands.

4. We have successfully recovered 7 cDNA molecules from 11 candidates by PCR and have their sequences analyzed.

5. We have successfully examined the expression status of the 7 candidate in a large number of PINs.

6. We have studied the prognostic significance of the OPN on survival of prostate cancer patients.

Reportable Outcomes

Shiva S. Forootan, Christopher S. Foster, Vijay R. Aachi, Janet Adamson, Paul H. Smith, Ke Lin, and Youqiang Ke. Prognostic significance of osteopontin expression in human prostate cancer. Int. J. Cancer 2005, in press.

Conclusions

Our main purpose in this explorative project is to collect some preliminary data to enable us to write a full project grant application to continue our study to identify the possible molecular markers to predict the malignant tendency of the cancer-precursor PIN lesions. During the past year, we have completed all proposed investigations. Our results showed that the strategy proposed in the original application is feasible to carry out and it is very likely the new markers that capable of predicting malignant tendency of PIN will be identified if this study is to be continued as the way proposed in the original plan.

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Appendice

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Prognostic significance of osteopontin expression in human prostate cancer

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To test the hypothesis that expression of osteopontin (OPN), an integrin-binding glycoprotein, can independently predict the potential aggressiveness of prostate cancer, the status of OPN expression in benign and malignant prostate cancer cell lines and tissues was analysed by Western blot and immunohistochemistry. Amongst the four prostate cell lines analysed, the level of OPN expressed in the benign PNT-2 cells was set at 1, the relative level of OPN expressed in the weakly malignant cell line LNCaP was increased to 1.5. In the highly malignant cell lines Du-145 and PC-3, the level of OPN expression was further increased to 2.9 and 4.4, respectively. An increased expression of OPN was also observed in the prostate tissue samples. When the level of OPN in normal tissue was set at 1, its level in benign prostate hyperplasia (BPH) was similar at 0.99 ± 0.2, whereas the OPN level in the highly malignant carcinoma tissue was greatly increased by nearly 6-fold to 5.9 ± 0.3. Amongst the 116 cases examined immunocytochemically, of the 10 normal cases, 3 (30%) were unstained and 7 (70%) stained weakly positive (+). Amongst the 36 BPH samples, 32 (89%) stained weakly positive (+) and 4 (11%) were unstained (−). For the 70 carcinomas analysed, 31 (44%) stained strongly positive (+++), 20 (29%) stained moderately positive (++) and 19 (27%) stained weakly positive (+). These results showed that the level of OPN expressed between the normal and the BPH samples was not significantly different (Fisher's exact test, $p = 0.16$). However, in comparison to that in the BPH samples, the expression of OPN in the carcinoma tissues was significantly increased (Chi-square test, $p < 0.0001$). Kaplan-Meier survival analysis showed that the increased level of OPN expression was significantly ($n = 70$, $p = 0.03$) associated with reduced survival time of the patients. The OPN expression was increased with the increasing Gleason scores of the carcinomas (Chi-square test, $p < 0.001$). The results in our study support our hypothesis and suggest that the increased OPN level may be involved in the malignant transformation of prostate epithelial cells and OPN expression level is an important determinant for patient survival.

Key words: prostate carcinoma; osteopontin; benign prostate hyperplasia (BPH); Gleason scores; patient survival

Prostate cancer has become the most common male cancer and the second leading cause of death from male malignant disease in the United States and Europe, and this disease kills more than 40,000 men per year in the developed world.[1,2] Despite the increasing incidence, the molecular basis of prostate cancer progression, invasion and metastasis is still not fully understood. The complicated molecular pathology of prostate cancer may involve a series of sequential genetic events, which cause the initiation and progression of the malignant changes of prostate epithelial cells. The complicated genetic events responsible for tumorigenicity and metastasis of prostate cancer may be classified in a simple manner as the increased activity of the possible cancer-promoting genes (oncogenes) and the diminished activity of the possible cancer-suppressor genes (tumour suppressor genes).[2,3] Thus, identification of these oncogenes and tumour suppressor genes and determination of their potential significance in prognosis, diagnosis and treatment are of paramount importance in prostate cancer research.

Osteopontin (OPN), an integrin-binding glycoprotein, is a promoting factor for tumorigenicity and metastasis of cancer cells in breast, lung, thyroid and liver through a variety of mechanisms.[4–9] High level of OPN expression in breast cancer is significantly associated with the early demise of the breast cancer patients (Rudland *et al*. 2002). Despite the previous studies, the expression status and the role of OPN in prostate cancer were not completely clear. It is not understood whether the high level of OPN expression in prostate cancer is significantly associated with the early demise of the patients, as seen in those of breast cancer (Rudland *et al*. 2002). A previous study suggested that OPN was over expressed in human prostate cancer tissue and that OPN can stimulate the growth of weakly malignant prostate cancer cells, suggesting that OPN might play a role in prostate cancer progression.[10,11] However, this result was disputed by the finding that the difference in OPN expression between the carcinoma and benign prostate hyperplasia (BPH) tissues was too small to be significant and thus, although OPN may be used as a indicator for differentiation, it cannot be used as a marker of malignancy.[12] The discrepancy between these studies has provided impetus to clarify the OPN expression status and its prognostic potential in a large number of prostate tissues.

In our work, we have systematically examined the expression status of OPN in benign and malignant prostate epithelial cell lines and in an archival set of a large number of prostate tissues. The levels of OPN expression have been analysed in different cultured cell lines and different types of tissues to assess whether the increased OPN is related to the malignant characteristics of the cells. The intensity of immunohistochemical staining amongst the normal, BPH and malignant carcinoma tissues has been compared so as to decide whether OPN is overexpressed in prostate carcinomas. The staining intensities of the tissue samples of low (Gleason scores 2–4), moderate (Gleason scores 5–7) and highly (Gleason scores 8–10) malignant carcinomas have also been compared to assess whether the high level of OPN expression is related to the degree of malignancy. More importantly, the relationship between the OPN expression and the survival time of the prostate cancer patients has been assessed by Kaplan-Meier survival analysis. The relationship between the patient survival and the Gleason scores (GS) of the carcinomas has also been assessed and the prognostic significance of OPN expression is compared with that of the GS.

Material and methods

Cell lines and culture conditions

Four human prostate cell lines were used in this work: the benign prostate epithelial cell line PNT-2,[13,14] the weakly malignant cell line LNCaP[15] and the highly malignant cell lines Du-145[16] and PC-3.[17] The cell lines were grown and maintained as monolayer cultures in RPMI 1640 (Invitogen, Paisley, Scotland) supplemented with 10% (v/v) FCS (Biosera, East Sussex, UK), penicillin (100 units/ml) and streptomycin (100 μg/ml) (Invitrogen). For LNCaP cells, hydrocortisone and testosterone (50 ng/ml) (Sigma, Gillingham, UK) were added to the culture medium.

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Detection of OPN protein in cultured cells and tissues

The OPN expression in different cell lines and tissues was detected by Western blotting, using a ECL light-emitting non-radioactive kit (Amersham Pharmacia Biotech., UK) in a similar way as described previously.[18] The total protein in each sample (20 μg) was quantified with a Coomassie Protein Assay Reagent kit (Pierce, IL, USA). Cell extracts prepared from different cell lines were subjected to SDS-PAGE in 10% (w/v) polyacrylamide gels. A recombinant OPN protein sample was loaded on the left side of the gel to act as a size marker. The separated proteins were transferred onto a nitrocellulose membrane (Hybond, Amersham Biosciences, UK) at 100 V for 2 hr under cold condition (4°C). The membrane was first incubated with preblocking reagents and then incubated with anti-human OPN MAb (Developmental Studies Hybridoma Bank, IA, USA) with 1/250 dilution of 3.8 mg/ml stock solution overnight at 4°C. The membrane was then incubated with horseradish peroxides-conjugated rabbit anti-mouse antibody (DAKO, UK) and bound antibodies were revealed by chemiluminescence (ECL, Amersham BioTech) and recorded on Kodak XAR-5 film. The intensities of OPN bands were determined by scanning densitometry, using Alphalmager 2000 software (Alpha Innotech, Cannock, UK). The values of the bands were obtained by measuring the areas of the peaks. The relative levels of OPN expression in different malignant cell lines and different types of tissues were determined by comparing the intensities of the bands with that of the benign cell line PNT-2 or the normal tissue. To standardise the quantitative measurements, the blot was incubated with specific anti-human actin MAb (Sigma, UK), and the possible loading errors were corrected by relating the images of OPN bands to those of the β-actin bands. Results were expressed as the mean ± SD of 4 separate measurements.

Human prostate tissues

The human prostatic tissues were an archival set with up-to-date- follow- up data held within the surgical pathology archive in the Department of Pathology, University of Liverpool, UK. Tissues were taken from 70 prostate adenocarcinoma patients with a mean age of 73 years and from 36 BPH patients with a mean age of 67.5 years, through Trans-Urethral Resection (TUR) in Royal Liverpool University Hospital during the 5-year period of 1995–2000. The 10 normal prostate tissues were taken from road accident victims who did not have a history of any prostatic diseases. Our study was approved by the Liverpool University Science Ethics Committee, in accordance with the Medical Research Council guidelines (project reference number: 02/019). Histological sections (4 μm) of formalin-fixed and paraffin-embedded tissues were cut and processed, as described previously.[19] The tissue sections were examined independently by 2 qualified pathologists and classified as normal, BPH and carcinomas. The carcinomas were further classified according to their combined GS.[20]

Immunocytochemical detection of OPN protein in prostate tissues

Immunohistochemical staining was used to detect the OPN expression in human prostate tissues. The details of the procedures for tissue staining were similar to those described previously.[21] After deparafinisation in xylene and rehydration in ethanol, the sections were immersed in methanol containing 3% hydrogen peroxide for 12 min followed by rinsing in tap water and immersing in distilled water. The slides were then incubated with anti-OPN monoclonal antibody MBIII B10 (Developmental Studies Hybridoma Bank, IA, USA) at a dilution of 1/500 of 3.8 mg/ml original stock solution overnight at 4°C. The bound antibodies were detected with 200 μl of reagents from Envision System Horseradish Peroxides kit (Dakocytomation, Ely, UK) for 30 min and the reaction was visualised with DAB (3-3′-diamono-benzidine) for 10 min. The cellular nuclei were counterstained with haematoxylin and the sections were mounted with DPX. To test its specificity, the antibody was incubated with 10 μg/ml of recombinant human OPN protein (R&D system, UK) at 4°C overnight, which completely blocked any immunolabelling. Intensity of staining was classified by the proportions of the cells stained. For each slide, 10 random microscopic fields were chosen and 100 cells were counted in each field. The percentage of the stained cells was decided by calculating the average of the 10 fields. Tissues stained with less than 1% of cells were classified as negative stain or unstained (−). Those with up to 30% of the cells stained were classified as weakly positive stain (+). Tissues stained 30–70% and more than 70% of cells were classified as moderately positive stain (++) and strongly positive stain (+++), respectively. One carcinoma sample with high GS was used as a positive control for each set of the experiments. The results were initially assessed by a qualified pathologist, then separately assessed by two other qualified pathologists and finally assessed by the 3 pathologists collectively. The final results were unanimously agreed amongst the 3 qualified pathologists.

Statistical analysis

The correlation between the OPN staining and the nature of the prostate tissues (normal, benign and malignant) was assessed by 2-sided Fisher's exact test and Chi-square analysis, using the Statistical Package for the Social Science Version 12.0 (SPSS Inc., Chicago, IL, USA). The correlation between OPN staining in prostate carcinoma and patient survival and also the correlation between GS and patient survival were evaluated by using the Kaplan-Meier method and differences between patients groups were assessed by the log rank test. The association between GS of the carcinomas and the OPN stains was assessed by Chi-square test. Statistical significance was defined as a $p < 0.05$.

Results

The level of OPN expression in prostate cell lines and tissues

Western blot analysis detected a single OPN band with a size of 64 kDa in all cell lines examined (Fig. 1*a*). Further quantitative measurements on OPN expression levels in different cell lines are shown in Figure 1*b*. The level of OPN expression detected in the benign PNT-2 cells was set at 1 and the relative level of OPN expressed in the weakly malignant cell line LNCaP was increased to 1.5 ± 0.3. In the highly malignant cell lines Du-145 and PC-3, the level of OPN expression was further increased to 2.9 ± 0.5 and 4.4 ± 0.6, respectively. F1

Similar pattern of OPN expression was detected in prostate tissues (Fig. 2*a*). When the level of OPN expressed in the normal prostate tissue sample was set at 1, as show in Figure 2*b*, its relative level of expression in the BHP was barely changed at 0.99 ± 0.2. However, the relative level of OPN expressed in the high malignant carcinoma tissue was dramatically increased by nearly 6-fold to 5.9 ± 0.3. F2

OPN expression in prostate tissues

Examples of the immunohistochemical stains of different prostate tissues with the OPN-specific monoclonal antibody (MAb) are shown in Figure 3. The area of stain in most of the tissues was located in the cytoplasm of the epithelial cells of prostatic glands with heterogeneous patterns. In some glands of normal and BPH tissues, weak staining was observed in basal cells, but this feature was not seen in carcinomas since basal cells are absent in this type of tissues. When the OPN-specific MAb was neutralized with the recombinant OPN protein, staining was blocked completely (Figs. 3*g* and 3*h*). The detailed results of OPN stain intensities in different prostate tissues are summarised in Table I. Of the total 116 cases examined, 3 (30%) of the 10 normal cases were unstained and the other 7 (70%) exhibited a weakly positive stain. Amongst the 36 BPH samples, 32 (89%) stained weakly positive and 4 (11%) were unstained. No significant difference (Fisher's exact test, $p = 0.16$) was observed between the staining patterns of normal prostate tissue and BPH. For the 70 carcinomas analysed, 31 (44%) stained strongly positive, 20 (29%) stained moderately F3 T1

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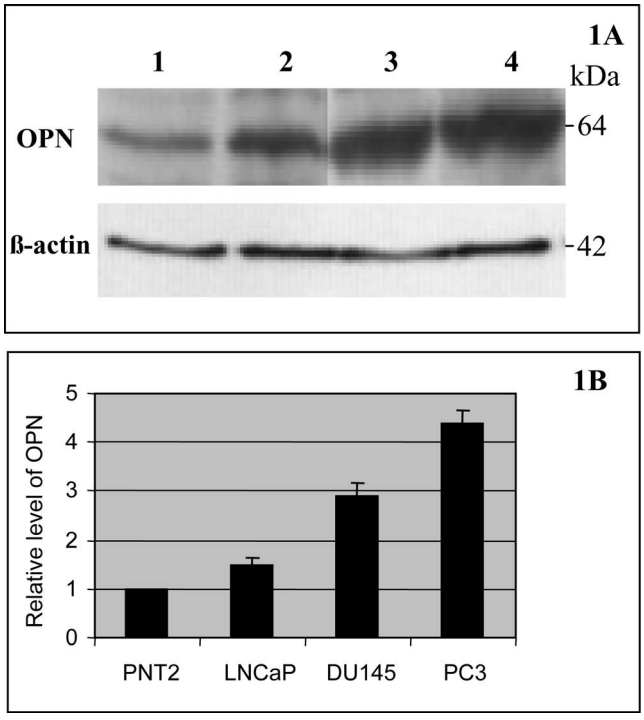

FIGURE 1 – Analysis of the levels of OPN expression in benign and malignant prostate cell lines. (*a*) Western blot analysis of OPN expression in different cell lines. Lane 1, the benign cell line PNT-2; Lane 2, the weakly malignant cell line LNCaP; Lane 3 and 4, the highly malignant cell lines Du-145 and PC-3. The antibody against β-actin was used to standardise the amount of proteins loaded in each sample on the same blot. (*b*) Relative level of OPN expression in different cell lines. The level of OPN expressed in benign PNT-2 cells was set at 1.0 and the relative levels of OPN expressed in other cell lines were obtained by comparing the expression values to the level of PNT-2. The discrepancies on loading were correlated by relating to the amount of β-actin. Results were the mean ± SD of 4 separate measurements.

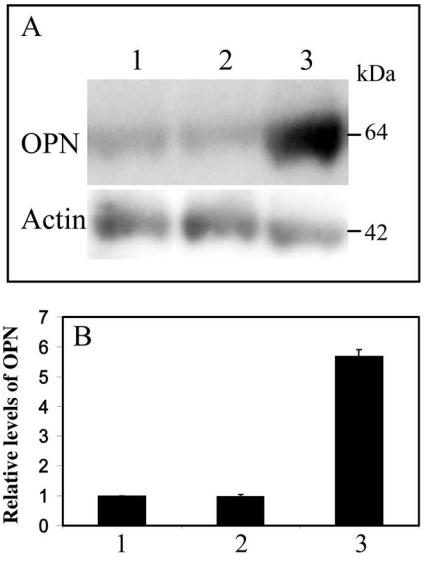

FIGURE 2 – Analysis of OPN expression in different types of prostate tissues. (*a*) Western blot analysis of OPN expression in normal, BHP and carcinoma tissues. Lane 1, normal prostate tissue; Lane 2, BPH; and Lane 3, a high malignant carcinoma with GS 10. The antibody against β-actin was used to standardise the amount of proteins loaded in each sample on the same blot. (*b*) Relative levels of OPN expressed in different types of tissues. The level of OPN expressed in normal tissue was set at 1.0 and the relative levels of OPN expressed in BPH and carcinoma tissues were obtained by comparing the expression values to the level of the normal tissue. The discrepancies on loading were correlated by relating to the amount of β-actin. Results were the mean ± SD of 4 separate measurements.

positive, 19 (27%) stained weakly positive and no samples were unstained (0%). The intensity of the staining in carcinomas is significantly stronger than that in normal (Chi-square test, $p < 0.001$) and in BPH (Chi-square test, $p < 0.001$) tissues.

OPN expression and GS

The relationship between the intensity of OPN staining in the 70 carcinomas and their GS is shown in Table I. Amongst the 20 weakly malignant carcinomas with GS 2–4, 17 (85%) stained weakly positive, 2 (10%) stained moderately positive and 1 (5%) stained strongly positive. Amongst the 30 moderately malignant carcinomas with GS 5–7, 4 (13%) stained weakly positive, 14 (47%) stained moderately positive and 12 (40%) stained strongly positive. The intensity of the stains in moderately malignant cases is significantly stronger than that observed in the weakly malignant cases (Chi-square test, $p < 0.001$). Of the 20 highly malignant carcinomas with GS 8–10, none was unstained (−) or stained weakly positive (+), two cases (10%) stained moderately positive (++) and 18 (90%) stained strongly positive (+++). The intensity of the stains in the highly malignant cases is significantly stronger than those observed in both the weakly malignant group (Chi-square test, $p < 0.001$) and the moderately malignant group (Chi-square test, $p < 0.01$).

OPN expression and patient survival

To assess the relationship between the OPN expression and patient survival, the 70 carcinomas were divided into weakly positive (+), moderately positive (++) and strongly positive (+++) groups, according to their OPN staining intensities. The relationship between the cumulative probability of surviving and the survival time after the diagnosis for the 3 groups of patients is shown in Figure 4. The mean expected survival time for patients with weakly positive OPN stains was 61.60 months (range 47.47–75.73). The mean survival time for patients with moderately positive OPN stains was reduced to 48.56 (34.84–62.27) months. For those patients with strong positive OPN stains, the mean survival time was further reduced to 38.47 (27.17–49.76) months. Overall survival of patients with different OPN stain intensities was significantly different (Log rank test, $p = 0.03$). When the groups were compared separately, the survival of patients in moderately OPN positive cases (34.84–62.27 months) was shorter than that in the weakly OPN positive cases, but the difference was not significant (Log rank test, $p = 0.26$). The survival of the patients in the strong OPN positive cases was significantly shorter than that in weakly OPN positive cases (Log rank test, $p = 0.008$) but the difference in survival between the strong and the moderate OPN positive cases was not significant (Log rank test, $p = 0.19$).

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Gleason scores and patient survival

To assess the relationship between the combined GS and patient survival, 70 carcinoma cases were divided into 3 groups, according to the different degrees of malignancy: the weakly malignant group with GS of 2–4, the moderately malignant group with GS of 5–7 and the highly malignant group with GS of 8–10. The mean survival of patients in the weakly malignant group was 66.20 months (54.07–78.33 months). The mean survival of patients in the moderately malignant and the highly malignant groups was 48.24 months (37.33–56.15 months) and 31.42 months (17.99–44.85 months), respectively. The relationship between the cumulative probability of surviving and the survival time after the diagno-

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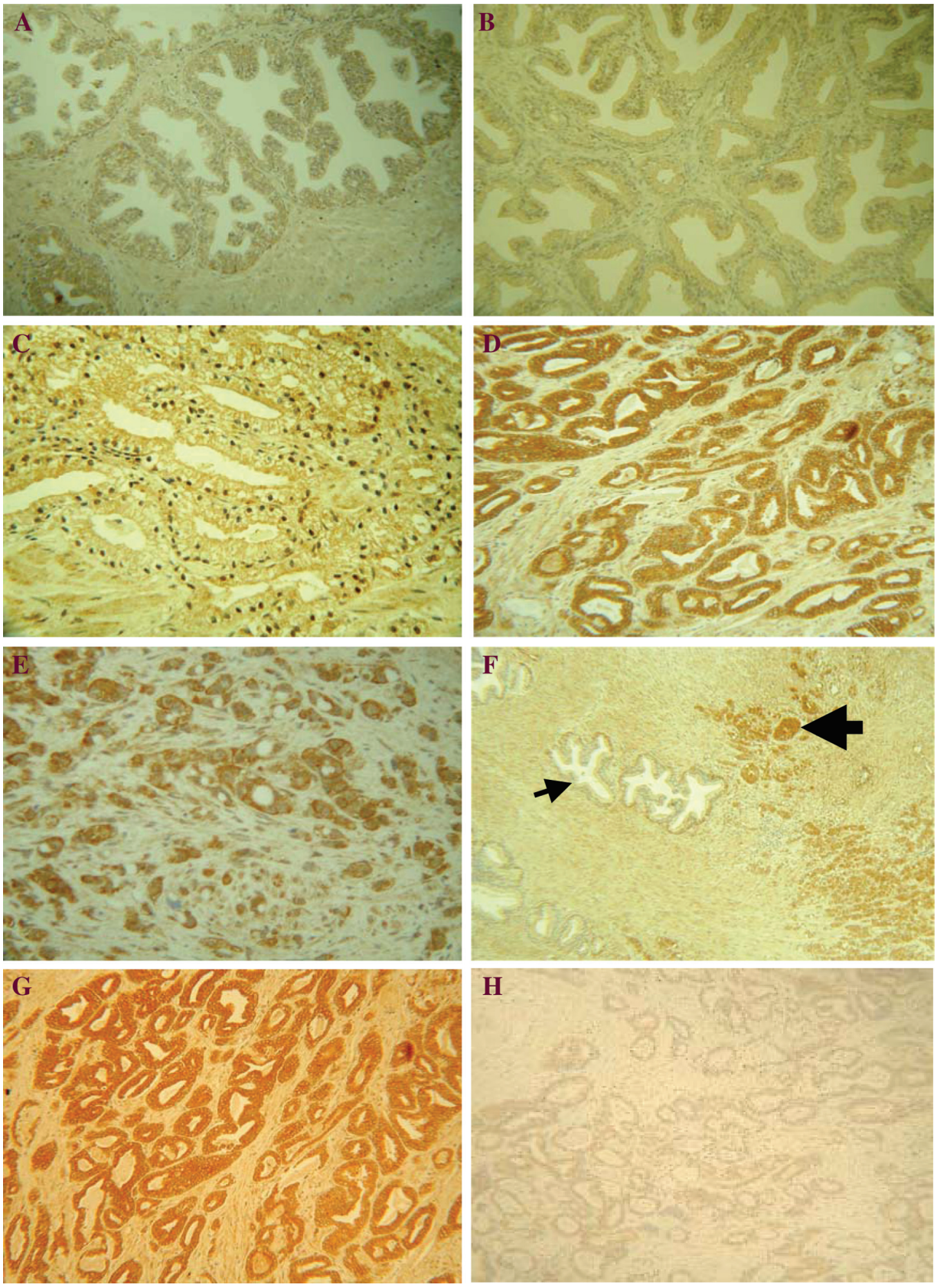

COLOR

FIGURE 3.

TABLE I – OPN EXPRESSION IN DIFFERENT PROSTATE TISSUES

Tissues	Stain intensities				No. of cases
	–	+	++	+++	
Normal	3	7	0	0	10
BPH	4	32	0	0	36
Carcinomas (total)	0	19	20	31	70
GS 2–4	0	17	2	1	20
GS 5–7	0	4	14	12	30
GS 8–10	0	0	2	18	20

F5 sis for these 3 groups of patients was shown in Figure 5. When GS were correlated with the patient survival, the survival time decreased with increased GS (Log rank test, $p = 0.001$). There was a highly significant difference between patients of highly malignant group and those of weakly malignant group in the length of the survival time (Log rank test, $P = 0.003$). The difference in survival time between the highly malignant cases and the moderately malignant cases was significant (Log rank test, $p = 0.02$), but difference between the weakly malignant cases and the moderately malignant cases was not significant (Log rank test, $p = 0.09$).

Discussion

Prostate cancer is characterized by clinical and biologic heterogeneity with complicated molecular and epidemiological dimensions. Despite the trend that prostate cancer is increasingly becoming a worldwide health threat, there are difficulties in its accurate diagnosis, prognosis and assessment of treatment effectiveness partially because of the lack of reliable biological markers to predict the malignant progression of the cancer cells. In recent years, great effort has been made to identify and isolate the genetic factors involved in the malignant progression of prostate cancer. Several new oncogenes and tumour suppressor genes had been discovered.[22–26] Identification of these genes and investigations on how they work within the cancer cells have greatly advanced our understanding of the complicated molecular pathology of prostate cancer and have also provided potential new targets for effective treatment. Although these genes had been shown to be important in the development and metastasis of prostate cancer, it is not clear whether they can be used as biomarkers to predict the malignant tendencies and the outcome of the patients. The routinely used prostate cancer marker Prostate Specific Antigen (PSA) is a very useful tool to screen prostatic diseases, but it can be affected by many other factors[27] and moreover, it merely reflect the prostatic volume. Although it is a good indictor of the size changes in the prostate gland, PSA alone cannot accurately predict the nature of the prostate cells, nor distinguish between the benign and malignant status. Therefore, it is important to assess the prognostic potential of the currently identified metastasis-related genes.

One of the metastasis-related genes is the integrin-binding glycoprotein, OPN. This protein is expressed and secreted by numerous human cancers. The molecular mechanisms that define the role of OPN in tumour metastasis are incompletely understood. OPN functions in cell adhesion, chemotaxis, macrophage-directed interleukin-10 suppression, stress-dependent angiogenesis, prevention of apotosis and anchorage-independent growth of tumour cells by regulating cell-matrix interactions and cellular signalling through binding with integrin and CD 44 receptors.[28] It is reported that the expression of OPN in prostate cancer is accompanied by a sustained activation of epidermal growth factor receptor (EGFR), suggesting that a association of cell surface receptors such as the colocalisation of integrin β1, a ligand of OPN, and of EGFR on the cellular membrane may be a principle mechanism involved in malignant progression caused by long-term activation of the EGFR.[29]

Although OPN was expressed in all 4 cell lines examined in our study (Fig. 1*a*), the level of OPN in the weakly malignant cell line LNCaP was 1.5-fold higher than that detected in the benign PNT-2 cells. A further increase of 2.9- and 4.4–fold, respectively, was found in the highly malignant cell lines Du-145 and PC-3. Previous invasion assays have demonstrated the benign nature of PNT-2 cells and the progressively malignant characteristics of LNCaP, Du-145 and PC-3, in term of their ability invade basement membranes *in vitro*.[30,31] Western blot also detected a nearly 6-fold of increment of OPN expression in the highly malignant tissues when compared with those expressed in the normal and BPH samples (Fig. 2). Thus, in the prostate cell lines and tissues examined, the expression of OPN protein appears to be closely linked to the malignant potential of the cells. OPN may, therefore, be involved in malignant transformation and hence play an important role in pathogenesis of prostate cancer.

The immunohistochemical examination (Table I) showed that there was no significant difference (Fisher's exact test, $p = 0.16$) in OPN staining intensities between the normal and the BPH samples. However, amongst the carcinoma samples, the level of OPN staining in carcinoma tissues was significantly stronger than those observed in both normal (Chi-square test, $p < 0.001$) and BPH (Chi-square test, $p < 0.001$) tissues. This result suggests that the increase in OPN expression in prostate tissue is related to prostate cancer only, and not to benign prostatic conditions, indicating that the elevated OPN expression may be an important factor enabling malignant transformation of the prostate cells. Further study (Table I) on the correlation of the staining intensities and the degree of malignancy of the carcinoma tissues showed that the OPN intensity in moderately malignant prostate tissues were significantly (Chi-square test, $p < 0.001$) higher than that observed in the weakly malignant tissues. The OPN stain intensity observed in highly malignant prostate tissues was significantly higher than those observed in both the weakly (Chi-square test, $p < 0.001$) and moderately (Chi-square test, $p < 0.01$) malignant tissues. These results suggest that the elevated OPN expression is closely associated with increased GS of the carcinomas, indicating the increase in OPN expression may play an important role in malignant progression of prostate cancer. The fact that the increase in OPN staining intensity from weakly to moderately malignant samples is larger than that from moderately to highly malignant tissues, maybe due to a threshold value for OPN, which promotes the malignant progression of prostate cancer, and once this threshold value is surpassed, any further increase of GS is accompanied by a reduced increment of OPN expression. Further study is needed to find out the exact role of OPN in malignant progression of prostate carcinomas.

When the relationship between the OPN staining intensity and the patient survival (Fig. 4) was assessed, a general inverse trend between a reduction in patient survival and increasing OPN staining intensity was observed (Log rank test, $p = 0.03$). Thus, the level of immunostaining for OPN may be used as a tool in the diagnosis and prognosis of prostatic biopsies. When similar assessment was made to examine the relationship between the GS and patient survival, it was found (Fig. 5) that the combined GS are significantly related to the mean survival of the patients (Log rank test, $p = 0.001$).

FIGURE 3 – Detection of OPN expression in normal, benign and malignant prostate tissues by immunohistochemical staining. The magnification of each slide is shown in brackets. (*a*) Normal prostate exhibiting a weakly stain (×250). (*b*) A BPH sample showing a weakly positive stain (×250). (*c*) A weakly malignant carcinoma with GS 4 staining moderately positive (×100). (*d*) A moderately malignant carcinoma with GS 7 staining strongly positive (×40). (*e*) A highly malignant carcinoma with GS 10 staining strongly positive (×400). (*f*) A strongly positive stain was observed in malignant carcinoma (large arrow), whereas only a very weak stain (small arrow) was seen in the adjacent benign areas within the same tissue sample (×100). (*g*) and (*h*) are test stains for the antibody specificity: the moderately malignant carcinoma with GS 7 stained strongly positive (*g*), but when the recombinant OPN protein was added to neutralise the MAb in the immunohistochemical reaction, no stain (*h*) was observed on the same carcinoma sample (×100).

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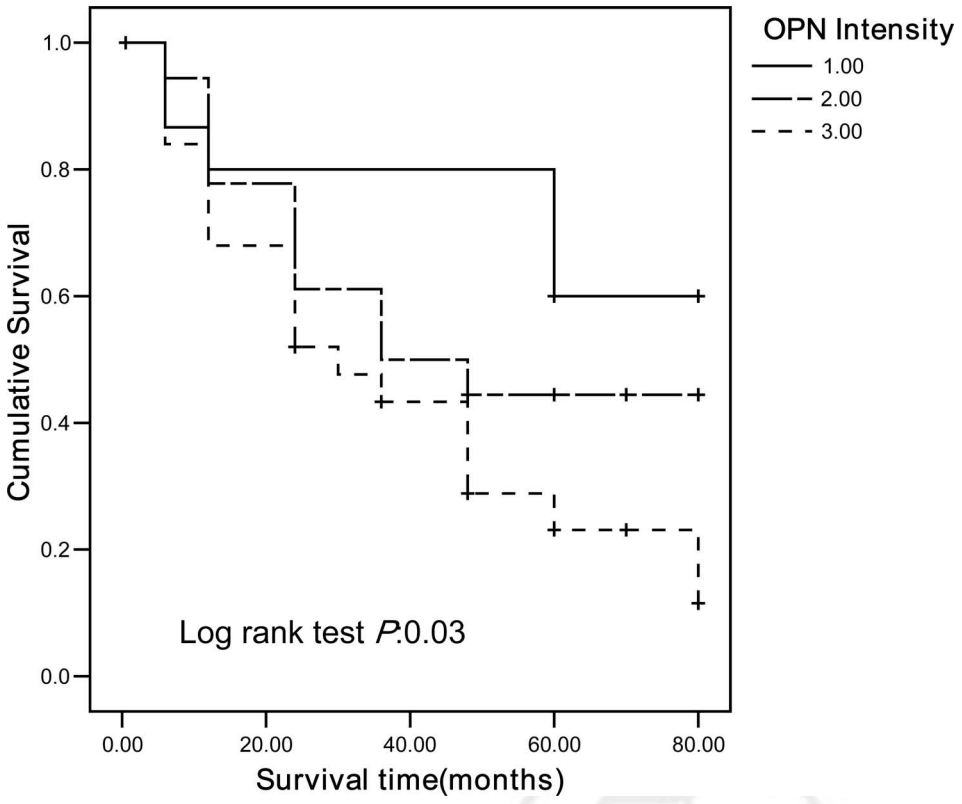

FIGURE 4 – Kaplan-Meier survival curves of 3 groups of prostate cancer patients with different OPN expression levels. Group 1, weakly positive OPN stains ($n = 19$); Group 2, moderately positive OPN stains ($n = 20$); and Group 3, strongly positive OPN stains ($n = 31$), as defined in the Material and Methods.

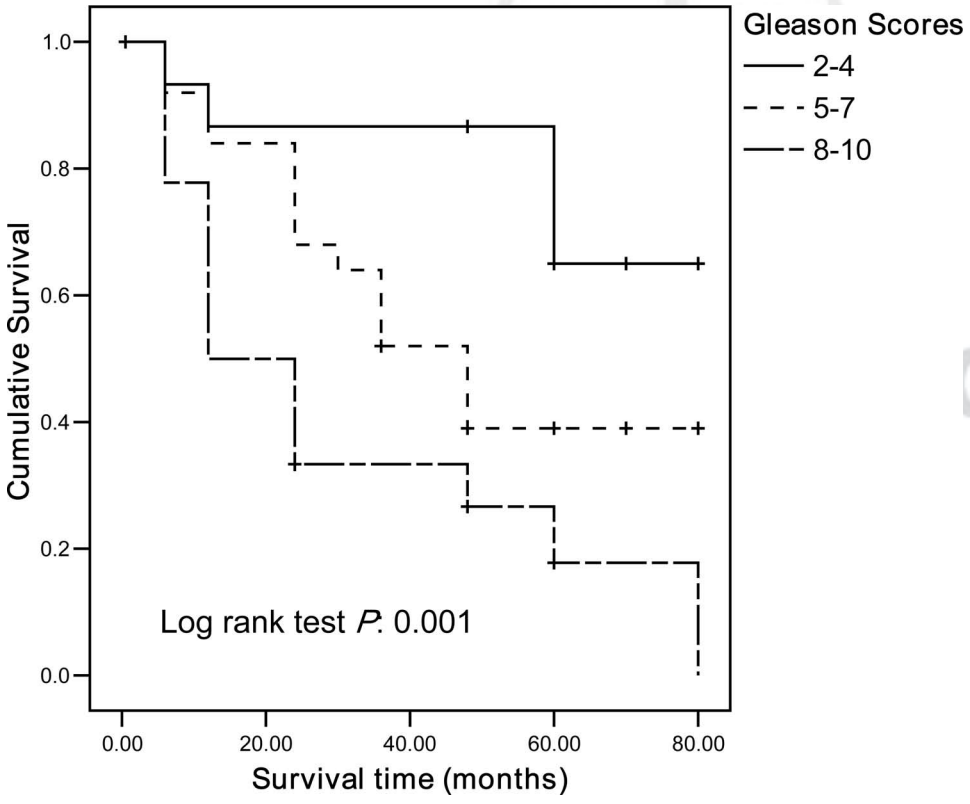

FIGURE 5 – Kaplan-Meier survival curves of 3 groups of prostate cancer patients with different combined GS. Group 1, GS 2–4 ($n = 20$); Group 2, GS 5–7 ($n = 30$); and group 3, GS 8–10 ($n = 20$).

The results in this work (Figs. 4 and 5) demonstrated that both Gleason score and the OPN staining intensities of the carcinomas were significantly associated with patient survival. Thus, both OPN stain and GS can be used as prognostic factors for prostate cancer patients. In comparison to the GS, OPN can be assessed through histological examinations, it also has a potential to be assessed by analysis of the blood and urine.[10,32] In addition, it should be easier to establish empirically applicable assays to quan-

tify OPN with techniques, such as Polymerase Chain Reaction (PCR).

In conclusion, OPN is expressed in higher levels in both malignant prostate cell lines and tissues compared with their benign counterparts. The increment of OPN is significantly associated with the increased malignant characteristics of the carcinoma cells. Moreover, the increased expression of OPN is significantly correlated with the reduced survival of the patients and both OPN staining and GS have a similar prognostic significance. Therefore, we suggest OPN may play an important role in the malignant progression of prostate cancer and it is a potentially useful prognostic factor to predict the outcome of patients with prostate cancer.

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